Imaging, Diagnosis, Prognosis

Identification of microRNA-135b in Stool as a Potential Noninvasive Biomarker for Colorectal Cancer and Adenoma

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Abstract

Purpose: Detecting microRNA (miRNA) in stool is a novel approach for colorectal cancer (CRC) screening. This study aimed to identify stool-based miRNA as noninvasive biomarkers for detection of CRC and adenoma.

Experimental Design: A miRNA expression array covering 667 human miRNAs was performed on five pairs of CRC and two pairs of advanced adenoma tissues. The most upregulated miRNAs were validated in 40 pairs of CRC tissues, 16 pairs of advanced adenoma tissues, and 424 stool samples, including 104 CRCs, 169 adenomas, 42 inflammatory bowel diseases (IBD), and 109 healthy controls. miRNA levels were followed-up after removal of lesions.

Results: In an array analysis, miR-31 and miR-135b were the most upregulated miRNAs in CRC and advanced adenoma as compared with their adjacent normal tissues (>13-fold increase). In stool samples, level of miR-135b was significantly higher in subjects with CRC ($P < 0.0001$) or adenomas ($P < 0.0001$), but not in patients with IBD compared with controls. miR-135b showed a significant increasing trend across the adenoma to cancer sequence ($P < 0.0001$). Levels of miR-31 were not significantly different among groups. The sensitivity of stool mR-135b was 78% for CRC, 73% for advanced adenoma, and 65% for any adenoma, respectively, with a specificity of 68%. No significant difference in the miR-135b level was found between proximal and distal colorectal lesions. Stool miR-135b dropped significantly upon removal of CRC or advanced adenoma ($P < 0.0001$).

Conclusion: Stool-based miR-135b can be used as a noninvasive biomarker for the detection of CRC and advanced adenoma. *Clin Cancer Res; 20(11); 2994–3002. ©2014 AACR.*

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide (1). Although the incidence of CRC is declining in the West, it remains the second most common overall cause of cancer death. Both the incidence and death rates from CRC are increasing rapidly in Asian countries (2). CRC screening allows the detection and removal of early-stage lesions, and has been demonstrated to reduce both CRC morbidity and mortality (3, 4). However, mass screening efforts have been hindered by variable public acceptance and the limitations of existing tools. Colonoscopy, although considered a gold-standard test for CRC screening, is associated with high cost and relatively low patient acceptance rate. The fecal occult blood test (FOBT) is the most widely adopted screening method, but is compromised by a low sensitivity and specificity and poor patient adherence. The newer fecal immunochemical tests (FIT) have demonstrated a higher sensitivity for CRC, but sensitivity remains low for premalignant precursor lesions (5). CT colonography is less invasive and accurate for the detection of CRC and advanced adenomas but is associated with high cost (6–9). Therefore, there is a need to develop better screening tools to avoid nontherapeutic colonoscopies for adenomas or colonoscopies to confirm CRC.

Testing for molecular aberrations in the stool has emerged as a promising noninvasive approach for CRC screening. Among stool-based molecular tests, DNA testing is the most established test (10–12). A DNA panel which combined four methylation markers, seven reference mutations, β-actin, and a hemoglobin assay, achieved a sensitivity

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Translational Relevance

Colorectal cancer (CRC) screening allows the detection and removal of early-stage lesions, and has been demonstrated to reduce both CRC morbidity and mortality. Compared with DNA, microRNA (miRNA) represents an emerging class of biomolecule being utilized as stool-based marker for CRC screening. In the current study, we demonstrated for the first time, in a large cohort of patients with CRC or advanced adenoma that miRNA (miR-135b), elevated in tumor tissue and stool samples, can be used as stool-based biomarker for CRC as well as adenomas. We revealed that the detection of miR-135b in stool has a sensitivity of 78% for CRC and 73% for advanced adenoma, thus stool-based miR-135b can be used as a potential noninvasive biomarker for the diagnosis of CRC and adenoma.

of 85% for CRC and 54% for adenoma ≥ 1 cm. Each gene typically yielded an area under the curve (AUC) value ranging from 0.61 to 0.75 toward CRC.

microRNAs (miRNA) are short noncoding RNAs that regulate gene translation (13). Most tumor types, including CRC, are found to have altered miRNA expression profiles (14–17). As tumor cells shed from CRC tumor surface into the lumen, aberrantly expressed miRNA levels can be detecting the stool. We have previously demonstrated that stool miRNAs (miR-21 and miR-92a) are stable and reproducible in a small number of the samples using an optimized RNA extraction and quantitative reverse transcription PCR (qRT-PCR) protocol (18). This provides a rationale of utilizing stool miRNA for the detection of CRC and adenomas. So far, four other studies have reported the use of stool-based miRNA as a screening tool for CRC (19–22). Most of these studies involved small cohorts and were limited to the investigation of CRC.

In this study, we aimed to identify miRNA markers with higher sensitivity, particularly for the diagnosis of precancerous colorectal lesions in additional consecutive stool samples particularly from subjects with advanced adenomas. We investigated the miRNA expression profile in CRCs and in advanced adenomas to identify the most upregulated miRNAs. Candidate miRNA markers were validated in 40 paired primary CRC tumors and 16 paired advanced adenoma tissues, and then in a large cohort of 424 stool samples of 104 CRC, 169 adenomas, 42 inflammatory bowel disease (IBD) as disease control, and 109 healthy subjects as normal control. We identified and characterized that stool-based miR-135b accurately detects CRC and adenoma in this large case–control study.

Materials and Methods

Subjects and stool sample collection

Stool samples were collected from 424 subjects, including 104 patients with CRC (mean age, 66.8 ± 11.9 years), 59 patients with advanced adenomas (62.1 ± 9.5 years), 110 subjects with adenomas of less than 1 cm in size (58.9 ± 6.9 years), 42 subjects with IBD (48.2 ± 11.6 years), and 109 individuals who had a normal colonoscopy (60.4 ± 7.0 years; Table 1). We have included patients with IBD as a control group to demonstrate that the markers are specific to CRC and precancerous adenoma but not to common inflammatory intestinal diseases. The mean age of the CRC group was significantly older than the control group ($P <$ 0.0001), whereas subjects with IBD were significantly younger than the control group ($P < 0.0001$). There were more males in the CRC than control group (58% vs. 46%; $P <$ 0.0001; Table 1). Exclusion criteria included subjects with a family history of familial adenomatous polyposis or hereditary nonpolyposis CRC, previous colonic surgery, or adjuvant therapy for CRC before surgery. Patients who were passing liquid stool were also excluded (Supplementary Fig. S1). All patients were recruited from Prince of Wales Hospital and Alice Ho Miu Ling Nethersole Hospital (Hong Kong, China). All participants had signed informed consent for obtaining stool or tissue samples. The study protocol was approved by the Institutional Review Board of the Chinese University of Hong Kong and the Hong Kong Hospital Authority (Hong Kong, China).

All cancer stool samples were collected 7 days after colonoscopy. All normal and adenoma stool samples were collected before colonoscopy. Fresh human stool samples were collected from patients using a 30-mL universal sample container (height, 93 mm; cap diameter, 30.1 mm) with spoon cap. The container was aseptically manufactured under clean room condition to exclude microbiological contamination. All samples were stored at 4°C immediately and transferred to −80°C within 24 hours. Stools were collected before bowel purgation and colonoscopy or 1 week after colonoscopy (but before resection of CRC or removal of advanced adenoma).

To investigate the changes in stool miRNA levels after removal of CRC or advanced adenomas, repeat stool collection was performed at least one month after surgical removal of CRC or at least 7 days after removal of the advanced adenoma. Advanced adenomas were defined as adenomas ≥ 1 cm in diameter, adenomas with villous or tubullovillous features, or high-grade dysplasia. Proximal lesions included lesions at or proximal to the splenic flexure, and distal lesions were lesions distal to the splenic flexure.

Tissue collection

Forty pairs of CRC tissues and 16 pairs of advanced adenoma tissues were collected (Supplementary Table S1). CRC and advanced adenoma tissues and their respective adjacent normal tissues (at least 4 cm apart from the lesion) were biopsied during the initial colonoscopy or during surgical resection. "Paired lesions" refers to a lesion paired with adjacent normal mucosal sample. Tissue samples were snap frozen upon collection and stored in −80°C freezer.

miRNA extraction in tissue and stool samples

Frozen tissue of 10 to 20 μg was added into 0.5 mL TRIzol reagent (Invitrogen) in a 1.5-mL tube. The tissue was

Table 1. Clinicopathological characteristics of subjects

Category	Normal	Adenoma < 1 cm	Advanced adenoma[a]	CRC	IBD
No. of cases	109	110	59	104	42
Age at enrollment, y					
Mean $\pm$ SD	60.4 ± 7.0	58.9 ± 6.9	62.1 ± 9.5	66.8 ± 11.9	48.2 ± 11.6
Gender, number (%)					
Female	59 (54.1)	51 (46.4)	29 (49.2)	44 (42.3)	16 (38.1)
Location[b], number (%)					
Proximal	—	32 (29.1)	26 (44.1)	29 (27.9)	—
Distal	—	65 (59.1)	31 (52.5)	75 (72.1)	—
Both	—	13 (11.8)	2 (3.4)	0 (0.0)	—
Tumor histology, number (%)					
Adenocarcinoma	—	—	—	97 (93.3)	—
Mucinous adenocarcinoma	—	—	—	6 (5.8)	—
Signet ring cell and mucinous	—	—	—	1 (0.9)	—
TNM stage[c], number (%)					
I and II	—	—	—	24 (23.1)	—
III and IV	—	—	—	76 (73.1)	—

[a]Advanced adenoma is defined as adenoma 1 cm or greater in diameter, adenoma with more than 25% villous feature, or adenoma with high-grade dysplasia.
[b]Proximal lesions include tumors at or proximal to the splenic flexure, and distal lesions are those distal to the splenic flexure.
[c]TNM stage data of four patients were unavailable.

homogenized by RNase-free pestles. Chloroform of 200 μL was added to the 1.5 mL tube.

Fresh human stool sample (20–30 g) was collected with a 50-mL specimen cup and stored in −80°C. Four categories of stool consistency were defined: "firm" (the stool has clear-cut edges, maintains its own shape during handling but deforms with pressure), "soft" (the stool has a uniform consistency but few or less apparent natural edges, it maintains its own shape but deforms with minimal handling), "loose" (the stool has a semisolid consistency and can take over the shape of the container), "watery" (no solid pieces, completely liquid). Only "firm," "soft," and "loose" stool samples were analyzed. More than 90% stool samples in each group belong to soft or loose. Stool sample of 200 to 300 mg (wet weight) was added to 1 mL TRIzol LS reagent in a 2-mL tube (Invitrogen), and homogenized mechanically by RNase-free pestles (USA Scientific) to deform it completely. Chloroform of 300 μL was added to the 2 mL tube.

Total RNA was extracted from the TRIzol-chloroform mixture using the miRNeasy Mini Kit (Qiagen). Total RNA was eluted in 50 μL nuclease-free water. RNA concentration was measured by Nanodrop 2000 (Thermo Fisher Scientific; the range of the initial RNA concentration RNA concentration was 300 to 500 ng/μL).

Reverse transcription and miRNA microarray in CRC and advanced adenoma tissues

RT for miRNA microarray was carried out using Megaplex Primer pools, Human Pools A and B v2.1 Kit (Applied Biosystems). Briefly, 2 ng total RNA was used in one RT reaction with a total volume of 3 μL. The RT product was diluted 4-fold by adding 9 μL nuclease-free water (18).

Initial miRNA profiling was performed on 14 tissue samples from five pairs of CRC and two pairs of advanced adenoma. The characteristics of these patients are shown in Supplementary Table S2. Quantitation of 667 miRNAs in each of these samples was carried out using TaqMan Human MicroRNA Array Set version 2.0 (Applied Biosystems). In the array, miRNAs were normalized to mammalian U6 small RNA expression as based on the manufacturer's guide (Applied Biosystems). qRT-PCR was performed using Applied Biosystems 7900HT Real-Time PCR System. Results were analyzed by the SDS RQ Manager 1.2 software (Applied Biosystems; Supplementary Table S3).

miRNA quantitation by qRT-PCR

qRT-PCR of individual miRNA was performed using TaqMan miRNA Reverse Transcription Kit (Applied Biosystems) and TaqMan Human MiRNA Assay (Assay ID: RNU6B, 001093; miR-31,002279; miR-135b, 002261). The quantitation of miRNA was based on standard curve plotted by known amount of synthetic miRNA, and normalized to per nanogram (ng) of input RNA (18). Assays were performed in a blinded fashion. On the basis of standard curves plotted from known amount of synthetic miR-135b, a technical detection limit of three copies of miR-135b would approximately give a C_t of 42. Therefore, we assigned all C_t larger than 42 as "0." For samples with no amplification of miR-135b at all, as long as that sample could be amplified with at least another miRNA tested (such as miR-18a, miR-20a, and miR-221), the sample was regarded to

have legitimate quality for qRT-PCR. Therefore, instead of excluding unamplifiable samples, we assigned the sample as "0" in the analysis of miR-135b.

Sample size and statistics

Given the exploratory aspect of this initial miRNA profiling and limitations of resources, we did not use a formal statistical test to choose a sample size, and we analyzed five pairs of CRC samples and two pairs of advanced adenoma sample for initial miRNA profiling. In our validation study, we analyzed 40 pairs of CRCs and 16 pairs of advanced adenoma tissue samples. Differences in miRNA expression between paired lesion tissues and adjacent normal tissues were evaluated by the Wilcoxon matched pair test. Differences in stool miRNA levels between groups were analyzed by the Mann–Whitney U test. Receiver operating characteristics (ROC) curves were generated on the basis of the comparison with the control group. Differences in miRNA levels before and after removal of the CRC or advanced adenoma were determined by the Wilcoxon matched pair test. Significance in trend was tested by the Jonckheere–Terpstra test. Two cutoff values were selected using ROC curves for reference, based on a high sensitivity or a high specificity. $P < 0.05$ was taken as statistically significant. The

Jonckheere–Terpstra test was done by SPSS 13.0 (SPSS Inc.). All other statistical tests were performed using GraphPad Prism 5.0 (GraphPad Software Inc.).

Results

miRNA profiling in CRC and advanced adenoma tissues

miRNA expression profiles were performed in five pairs of CRC samples and two pairs of advanced adenoma samples. The miRNA expression levels were compared between the tumor and their adjacent nontumorous tissues in each case (Table 2). Amongst 667 miRNAs detected in each sample, miR-31 and miR-135b were identified to be the most upregulated miRNAs in both CRC (miR-31, 42.28-fold increase; miR-135b, 13.00-fold increase) and advanced adenomas (miR-31, 106.36-fold; miR-135b, 13.32-fold; Table 2). We have therefore focused on these two miRNA in subsequent experiments.

Validation of miRNA candidates in CRC and advanced adenoma tissues

In a validation set of 40 pairs of CRCs and 16 pairs of advanced adenoma tissue samples, miR-135b was demonstrated to be 555.4-fold higher in CRCs ($P < 0.0001$) and

Table 2. Top 10 most upregulated miRNAs identified by profiling of 667 miRNAs in five patients with CRC and two patients with advanced adenoma

Rank	miRNA	Accession number	Chromosomal location	Average fold increase[a]
miRNAs with highest expression in colorectal tumor				
1	miR-31	MI0000089	9p21.3	42.28
2	miR-135b	MI0000810	1q32.1	13.00
3	miR-224	MI0000301	Xq28	5.48
4	miR-409-3p	MIMAT0001639	14q32.31	4.67
5	miR-18a	MI0000072	13q31.3	4.46
6	miR-452	MI0001733	Xq28	4.16
7	miR-221	MI0000298	Xp11.3	3.95
8	miR-21	MI0000077	17q23.1	3.39
9	miR-223	MI0000300	Xq12	3.18
10	miR-20a	MI0000076	13q31.3	3.02
miRNAs with highest expression in advanced adenoma				
1	miR-31	MI0000089	9p21.3	106.36
2	miR-135b	MI0000810	1q32.1	13.32
3	miR-20a-3p	MIMAT0004493	13q31.3	4.30
4	miR-182	MI0000272	7q32.2	3.99
5	miR-649	MI0003664	22q11.21	3.93
6	miR-26a-1-3p	MIMAT0004499	3p22.2	3.33
7	miR-625	MI0003639	14q23.3	3.13
8	miR-18a	MI0000072	13q31.3	2.78
9	miR-20a	MI0000076	13q31.3	2.75
10	miR-552	MI0003557	1p34.3	2.17

[a]Average fold change in colorectal tumor was expressed as ratio of the miRNA expression in tumor to adjacent nontumorous tissue. Average fold change in advanced adenoma was expressed as ratio of miRNA expression in advanced adenoma to adjacent nonadenomatous tissue.

Table 3. Expression of miR-135b and miR-31 in colorectal tumor or advanced adenoma

miRNA	Percentage of samples with elevated expression in tumor or advanced adenoma	Average fold increase	P^a
	CRC ($n = 40$)		
miR-135b	92.5% (37/40)	555.4	<0.0001
miR-31	87.5% (35/40)	105.1	<0.0001
	Advanced adenoma ($n = 16$)		
miR-135b	93.8% (15/16)	33.1	0.0003
miR-31	87.5% (14/16)	86.1	0.0003

$^a P$ values were estimated by the Wilcoxon matched pair test.

33.1-fold higher in advanced adenoma tissues ($P = 0.0003$), whereas miR-31 was 105.1-fold higher in CRCs ($P < 0.0001$) and 86.1-fold higher in advanced adenoma ($P = 0.0003$) compared with their corresponding normal tissues (Table 3).

miR-135b is a potential noninvasive stool marker for CRC and adenoma

We then examined the levels of miR-135b and miR-31 in 424 stool samples, which included 104 CRC, 169 adenomas, 42 IBD, and 109 controls. The stool-based miR-135b level measured in a number of copies/ng stool extracted RNA was significantly higher in subjects with CRC [median,

67.9, interquartile range (IQR), 16.1–182.7; $P < 0.0001$] and adenomas (median, 28.4; IQR, 0.2–79.7; $P < 0.0001$) compared with controls (median, 0; IQR, 0–30.8; Fig. 1A). In contrast, there was no significant difference in the level of stool miR-135b in subjects with IBD (median, 7.57; IQR, 0–60) compared with controls. The AUC values for miR-135b were 0.79 and 0.71 for the detection of CRC and adenomas, respectively (Fig. 1B). As shown in Table 4, two cutoff values demonstrated the performance of this marker: a cutoff of 14 copies/ng of stool RNA provided the maximum sum of sensitivity and specificity; miR-135b has a sensitivity of 78% for CRC, 73% for advanced adenoma, 61% for adenoma < 1 cm in diameter, 65% for any adenoma, and a specificity of 68%. A cutoff of 38 copies/ng of stool RNA reflects its performance at a relatively high specificity (80%) level for reference, and the sensitivity was 44%, 46%, and 64% for adenoma < 1 cm, advanced adenoma, and CRC, respectively (Table 4). However, there were no significant differences in the levels of stool miR-31 among CRC (median, 1,583; IQR, 574.5–3,364), adenomas (median, 1,647; IQR, 661.9–3,148), IBD (median, 1,642; IQR, 1,066–3,345), and controls (median, 1,293; IQR, 721–2,612; Fig. 1C and 1D).

Stool miR-135b level is significantly reduced after removal of neoplasm

We repeated miRNA measurement in a subgroup of 28 patients (8 CRC, 20 advanced adenomas) after the removal of CRC or advanced adenomas. Upon removal of the primary lesions, levels of stool miR-135b dropped significantly compared with their initial levels ($P < 0.0001$; Fig. 2A). These findings suggested that the initial high levels of stool miR-135b were derived from the primary neoplasms.
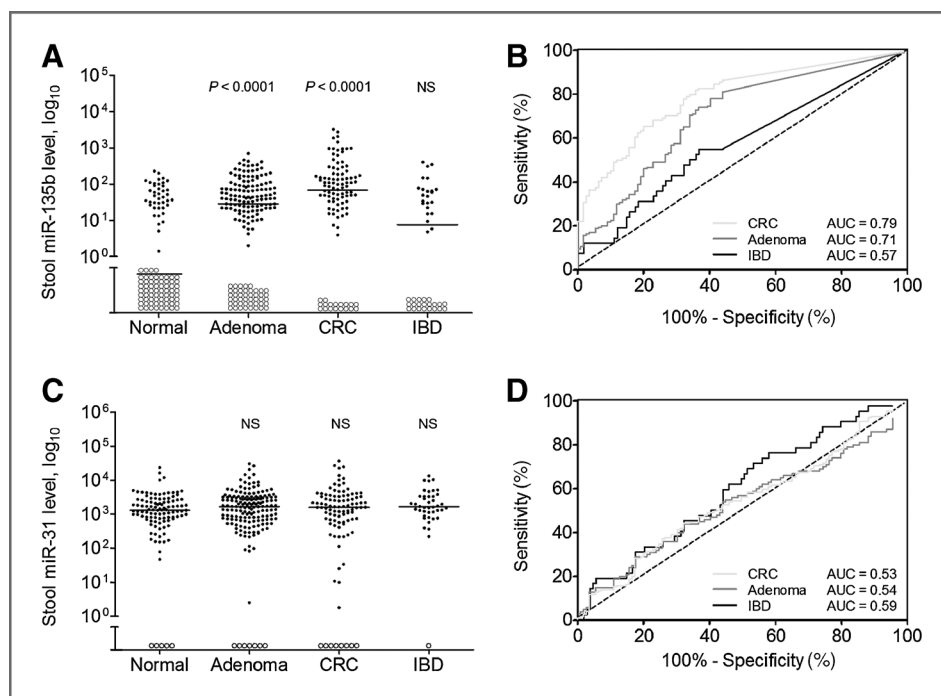
Figure 1. Levels of miRNA markers in stool samples. A, miR-135b and (B) its ROC curve, (C) miR-31 and (D) its ROC curve. Subjects were categorized into four groups: individuals of normal colonoscopy results ($n = 109$), adenoma ($n = 169$), CRC ($n = 104$), and IBD ($n = 42$), respectively. The miRNA levels were expressed in the number of copies per nanogram of extracted RNA. Open circles represent samples with undetectable miRNA level. The lines denote the medians. P denotes significance measured by the Mann–Whitney test. NS denotes no statistical significance. ROC curves were plotted to discriminate CRC, adenoma, or IBD patients from individuals with normal colonoscopy findings.

Table 4. Stool-based miR-135b sensitivities and specificities

Cutoff value[a]	14 (high sensitivity)	38 (high specificity)
Sensitivities, % (95% CI)		
Adenoma	65 (57–72)	45 (37–53)
Adenoma <1 cm	61 (51–70)	44 (34–53)
Advanced adenoma	73 (60–84)	46 (33–60)
Size		
1–1.9 cm	76 (55–91)	40 (21–61)
≥2 cm	83 (52–98)	75 (43–95)
Location		
Proximal	65 (44–83)	38 (20–59)
Distal	81 (63–93)	52 (33–70)
CRC	78 (69–85)	64 (54–74)
Stage		
TNM I, II	67 (45–84)	50 (29–71)
TNM III, IV	80 (70–89)	67 (55–77)
Location		
Proximal	79 (60–92)	59 (39–76)
Distal	77 (66–86)	67 (55–77)
Specificity, % (95% CI)	68 (58–77)	80 (71–87)

[a]Cutoff value was expressed as copies of the miRNA per nanogram of extract stool RNA. Two cutoff values were selected using ROC curves for reference.

Stool miR-135 level increases across the adenoma to cancer sequence

As shown in Fig. 2B, expression of stool miR-135 showed a significantly increasing trend across the disease transition from adenoma with diameter < 1 cm ($n = 110$), advanced adenoma ($n = 59$), tumor-node-metastasis (TNM) stage I and II cancer ($n = 24$) to TNM stage III

and IV cancer ($n = 76$) sequence ($P < 0.0001$). In keeping with this, an increasing sensitivity for stool-based miR-135 detection was demonstrated with 61% for adenomas with diameter < 1 cm, 73% for advanced adenoma, 67% for TNM stage I and II cancer, and 80% for TNM stage III and IV cancer, and a specificity of 58.4%. On the basis of Table 3, the increase fold change of miR-135b in CRC tissue samples is approximately 17-fold higher than in advanced adenoma tissue samples.

Stool miR-135 level is not associated with the location of CRC or advanced adenoma

We compared the level of stool miR-135 between distal and proximal lesions. No significant difference was found in miR-135b levels for CRC or advanced adenomas located in proximal or distal colon (Fig. 2C), although at a specific cutoff value (14 copies/ng RNA), miR-135b showed better sensitivity in detecting distal advanced adenoma (81%) compared with proximal ones (65%), with specificity of 68% for both distal and proximal advanced adenoma (Table 4).

Discussion

As CRC demonstrates high homogeneity in miRNA alteration, stool miRNA could represent a useful noninvasive tool for screening CRC and its precancerous lesions (18, 22, 23). For maximum benefit, a CRC screening tool should be effective in detecting CRC in its early stage and premalignant precursors throughout the entire colorectum. In this study, we performed systematical examinations with the aim to identify the best miRNA biomarker for the screening of CRC and advanced adenomas.

We first identified that miR-135b and miR-31 were the two most upregulated miRNAs both in CRCs and adenomas by miRNA expression arrays. In subsequent validation,
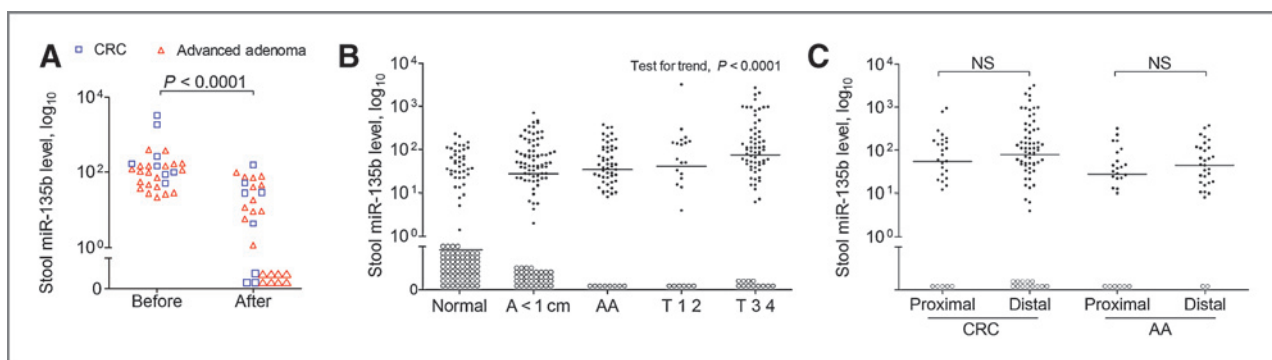
Figure 2. Associations between stool miR-135b and clinical features. A, changes of the stool miR-135b level after removal of tumors (squares) or advanced adenomas (triangles). Samples aligned on the x-axis represent those with undetectable miRNA level. P values indicate significant difference determined by the Wilcoxon matched pairs test. The stool miR-135b level in subjects categorized by lesion stage (B) and lesion location (C). On the basis of lesion stage, subjects were categorized into five groups: individuals of normal colonoscopy (normal, $n = 109$), patients with adenoma of diameter smaller than 1 cm (A < 1 cm, $n = 110$), patients with advanced adenoma (AA, $n = 59$), patients of tumor-node-metastasis (TNM) stages I and II (T 1 2, $n = 24$), and patients of TNM stages III and IV (T 3 4, $n = 76$), respectively. P value for test for trend denotes significance measured by the Jonckheere–Terpstra nonparametric test for trend. On the basis of lesion location, CRC, and advanced adenoma (AA) patients were categorized into proximal CRC ($n = 29$) and distal CRC ($n = 75$), proximal AA ($n = 26$) and distal AA ($n = 31$). NS denotes no statistical significance. miRNA levels were expressed in number of copies per nanogram of extracted RNA. Open circles represent samples with undetectable miRNA level. The lines denote the medians.

miR-135b and miR-31 were also found to be significantly upregulated in CRC and advanced adenoma as compared with their adjacent normal tissues (Table 3). This is consistent with previous studies showing that miR-135b (24, 25) and miR-31 (14, 25–28) are upregulated miRNAs in CRC. The levels of miR-135b and miR-31 were therefore tested in a large cohort of stool samples. Significantly higher level of miR-135b, but not miR-31, was detected in the stool samples of CRC ($P < 0.0001$) and advanced adenoma ($P < 0.0001$) compared with healthy controls (Fig. 1A). In addition, the miR-135b level correlated positively with stages of lesions, with more advanced lesions having the highest miRNA level. A significant increasing trend across the histologic sequence was observed from small adenoma, advanced adenomas, TNM stage I and II, to TNM stage III and IV ($P < 0.0001$; Fig. 2B). Areas under the ROC curve were 0.79 for the detection CRC and 0.71 for adenoma (Fig. 1B). At a cutoff of 14 copies/ng RNA, miR-135b had a sensitivity of 73% and 78% for the detection of advanced adenomas and CRC, respectively, with a specificity of 68%. We have included patients with IBD as a control group to demonstrate that the markers are specific to CRC and precancerous adenoma but not to common inflammatory intestinal diseases. The significant drop in the stool miR-135b level upon removal of advanced adenoma and CRC, and the relatively low level of miR-135b detected in stool of patients with IBD indicated that the upregulation of miR-135b is a specific biomarker for CRC and its precancerous lesion. In this study, we did not use RNU6B as internal control for stool miRNA detection. The choice and rationale of normalizing stool-based miRNA have been discussed in our previous study (18), which is mainly because RNU6B could only be detected in 83.3% (25/30) of stool samples, whereas the candidate miRNAs could be detected in 100% samples (18). In addition, under equal amount of input RNA and detection threshold, RNU6B was detected with a much lower abundance compared with candidate miRNAs (18). Thus, RNU6B may not be an ideal internal control for stool-based miRNA. In this regard, absolute quantitation with standard curve calibration was adopted for miRNA quantitation in the current study. Other upregulated miRNA candidates, including miR-18a, miR-20a, and miR-221 from, our miRNA array assay were also upregulated in CRC tissues compared with adjacent normal controls. As these miRNA candidates were not able to discriminate patients with adenoma from healthy individuals in stool samples (Supplementary Table S4), and the sensitivities and specificities of these miRNAs were lower compared with miR-135b for the stool detection, we did not include these miRNAs in this study. miR-135b was demonstrated to be the most discriminating marker for the detection of both advanced adenoma and CRC.

miR-135b originates from 1q32.1, a region of frequent copy number gain in CRC tumorigenesis. miR-135b targets the 3′untranslated region of the adenomatous polyposis coli (APC) gene, a well-known tumor suppressor, and suppresses its expression (24). APC downregulation activates the Wnt signaling pathway, an important oncogenic pathway in regulating cell proliferation and apoptosis in CRC. Upregulation of miR-135b is consistent with its role in regulating APC gene, whose loss-of-function is well established to be an early event of the adenoma-cancer sequence in colorectum.

Results from this study have several clinical implications. First, effective detection of proximal colon neoplasms is an important criterion for an effective screening tool (29). Current screening modalities seem less sensitive for proximal than distal colonic neoplasms, and interval CRC is more likely to be found in the proximal colon (11, 30). In this study, the miR-135b level has comparable efficacy for the detection of both proximal and distal CRC and advanced adenoma. Second, miR-135b represents a stool-based test that is noninvasive, avoids the unpleasant bowel preparation, and allows the possibility of off-site sample collection. Therefore, miR-135b may act as a potential noninvasive diagnostic biomarker for CRC and its precancerous lesion (advanced adenoma), but more studies in different populations to validate miR-135b as a biomarker for CRC screening are required before it can be applied clinically. Ultimately, test adoption will depend on test performance in the screening setting, availability, affordability, and user appeal in a large population-based program. For instance, screening using fecal DNA markers demonstrated a higher sensitivity than the FOBT, although the two tests yielded similar specificities (10). There has been no direct comparison of stool DNA versus RNA tests in the screening setting. The evidence for the effectiveness of gFOBTs in reducing CRC incidence and mortality is strong, and is based on randomized controlled trials in large number of average risk populations. FIT is a more sensitive test for screen-relevant neoplasia than FOBT; FITs processing and interpretation are automated and objective. Compared with gFOBT and FIT, miRNA lacks a large-scale validation. Only if this validation is conducted, the possible advantage of miRNA in detecting more proximal neoplasms than fecal blood tests will be known.

Stool miR-31 levels did not differ significantly among controls, adenoma, and CRC (Fig. 1C and 1D), and it did not correlate with the stool miR-135b level. We excluded the possibility that the aberrant upregulation of miR-31 was restricted to the submucosal regions given that the upregulation was also present in CRC epithelial cells (14). The reason why stool miR-31 is not discriminating remains to be elucidated; however, this demonstrates the practical fact that abnormalities at the tissue level do not necessarily translate into a clinically useful marker in the stool. As a noninvasive marker, empirical testing in large number of stool samples is required to establish its usefulness.

This study has several limitations. Most patients were recruited from only two centers and some patients were symptomatic, results might not be representative of the screening setting, although we did not detect significant difference in miRNA results when patients were divided into

asymptomatic screening or symptomatic surgical cohorts. More late-stage CRC patients were identified in the study ($n = 24$ for TNM I and II vs. $n = 76$ for TNM III and IV) and the stool samples from CRC used in the study might be biased to late stage. Prompted by these findings, larger scale validation across multiple centers and different populations will be conducted. In addition, there were more male subjects in the CRC group and they were significantly older than the control group. Nonetheless, when we took into account age and gender in the data analysis for the stool miR-135b level, our results remained valid and were independent of age and gender. We have not assessed the effect of time point of sampling (before vs. after colonoscopy) on miRNA results. In all patients with advanced adenomas, stool was collected one week before colonoscopy, whereas in subjects with CRC, stool samples were collected one week after colonoscopy but before surgery. Finally, there are no head to head comparisons of miRNA and FOBTs.

In conclusion, this study demonstrates that stool-based miR-135b seems to be a potential noninvasive biomarker for the detection of CRC and advanced adenoma. Further validation in multiple cohorts of patients and comparison with the established tests are needed before it can be used as a biomarker in routine clinical practice.

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Identification of microRNA-135b in Stool as a Potential Noninvasive Biomarker for Colorectal Cancer and Adenoma

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